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REGULAR ARTICLE

The inward-rectifying K^+ channel SsAKT1 is a candidate involved in K^+ uptake in the halophyte *Suaeda salsa* under saline condition

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Abstract

Background and aims The Shaker AKT1-like channels are considered to be involved in both high- and low-affinity K^+ uptake and correlated with salt tolerance in glycophytes. *Suaeda salsa* (*Suaeda maritima* subsp. *salsa*), as a typical salt-accumulating halophyte, is able to absorb K^+ efficiently while growing under saline conditions and taking in a large amount of Na^+, thus maintaining the K^+ concentration in its cells. In this study, the possible functions of the inward-rectifying K^+ channel SsAKT1 in K^+ uptake and salt tolerance in the halophyte *S. salsa* were investigated.

Methods SsAKT1 from *S. salsa* was isolated by RT-PCR and characterized using yeast complementation; the responses of *SsAKT1* to various KCl and NaCl treatments were investigated by real-time quantitative PCR.

Results SsAKT1 consisted of 879 amino acid residues and shared high homology (60–67 %) with the identified inward-rectifying K^+ channels AKT1 from other plants. The expression of *SsAKT1* rescued the K^+-uptake-defective phenotype of yeast strain CY162, and also suppressed the salt-sensitive phenotype of yeast strain G19, suggesting SsAKT1 functioned as an inward-rectifying K^+ channel. *SsAKT1* was predominantly expressed in roots, and was induced significantly by K^+ starvation; transcript levels increased further on resupply of K^+ (0.1–10 mM for 6 h) by 62 % in 0.1 mM K^+ and 144–174 % in higher K^+ concentrations (1–10 mM). Interestingly, the expression level of *SsAKT1* in roots was also induced significantly by short-term treatment (6 h) with NaCl concentrations (25–250 mM).

Conclusions These results demonstrate that the inward-rectifying K^+ channel SsAKT1 might mediate both high- and low-affinity K^+ uptake in *S. salsa*, but play a greater role in the low-affinity system. Furthermore, SsAKT1 might also be involved in salt tolerance by participating in the maintenance of K^+ nutrition in *S. salsa* under salinity.

Keywords *Suaeda maritima* subsp. *salsa* · AKT1 · Yeast complementation · K^+ uptake · Salt tolerance

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Introduction

Potassium (K^+) is an essential macronutrient for plant growth and development, accounting for 2–10 % of plant dry weight (Anschütz et al. 2014; Clarkson and Hanson 1980; Leigh and Wyn Jones 1984; Wang and Wu 2013). K^+ is the most abundant cation in the cytosol, playing crucial roles in many fundamental processes in plant cells, such as osmoregulation, regulation of membrane potential, electrical neutralization and serving as an activator of a large number of enzymes (Maathuis 2009; Römheld and Kirkby 2010; Véry et al. 2014). Salinity is a common cause of K^+ deficiency and is a serious factor limiting the productivity of agricultural crops (Kronzucker and Britto 2011; Munns 2002; Munns and Tester 2008; Zhang et al. 2010). However, halophytes have developed efficient mechanisms to adapt to highly saline environments during the process of long-term evolution (Bartels and Dinakar 2013; Flowers et al. 1977; Flowers and Colmer 2008, 2015; Shabala and Cuin 2008; Wang et al. 2002; Zhang and Shi 2013; Zhao et al. 2011), including the maintenance of internal K^+ concentrations.

The Amaranthaceae, *Suaeda salsa* (synonym of *S. maritima* subsp. *salsa*), a C_3 plant distributed in saline soil areas of northern China, has been paid much attention due to its economic and ecological value in saline agriculture (Li et al. 2011; Song et al. 2008; Zhao et al. 2002). *S. salsa* grows optimally in the presence of about 200 mM NaCl (Song and Wang 2014) and accumulates Na^+ to the concentration of about 400 mM based on the tissue water content in its leaves without injury, indicating that *S. salsa* is a typical salt-accumulating halophyte (Wang et al. 2004, 2007; Zhang et al. 2013). Although Na^+ has been shown to suppress K^+ influx (at both high- and low-affinity ranges, particularly in the low-affinity range at millimolar concentrations) in many plant species (Kronzucker et al. 2013), the selectivity for K^+ over Na^+ in *S. salsa* increased dramatically with an increase of NaCl concentration in medium, indicating that *S. salsa* was able to absorb K^+ effectively while taking in a large amount of Na^+ (Mori et al. 2011): under various NaCl treatments, K^+ absorption rate and the concentration of K^+ in whole plants of *S. salsa* was maintained at a relatively constant level (Mori et al. 2011). Thus, absorbing K^+ effectively and maintaining the stability of K^+ concentration in the plant might be key requirements for growth of *S. salsa* in highly saline soils.

In plants, K^+ acquisition from soils is mainly mediated by K^+ transporters and channels, such as those of the HKT family, HAK/KT/KUP family and shaker AKT1-like K^+ channels (Alemán et al. 2011; Mäser et al. 2001; Martinez-Cordero et al. 2005; Shabala 2003; Véry and Sentenac 2003; Véry et al. 2014; Wang and Wu 2013; Ward et al. 2009). Many HKT transporters in plants mostly function as Na^+ transporters, and only a few are Na^+ : K^+ symporters (Benito et al. 2014; Corratgé-Faillie et al. 2010; Gierth and Mäser 2007; Kronzucker and Britto 2011). Many HAK/KT/KUP transporters, which are sensitive to NH_4^+, have been reported as high-affinity K^+ transporters involved in K^+ uptake under K^+-deficient conditions (Elumalai et al. 2002; Gierth et al. 2005; Gierth and Mäser 2007; Grabov 2007; Nieves-Cordones et al. 2014; Pyo et al. 2010; Santa-María et al. 2000). The shaker AKT1-like channels, which are insensitive to high external NH_4^+ concentrations, are considered as the main channel components that mediate K^+ influx into root cells in many plant species (Chérel 2004; Fuchs et al. 2005; Hartje et al. 2000; Hirsch et al. 1998; Lagarde et al. 1996; Lebaudy et al. 2007). Shao et al. (2014) found that SsHKT1;1, a K^+ transporter from *S. salsa*, was involved in salt tolerance by taking part in cytosolic cation homeostasis, particularly affecting K^+ nutrition under salinity. Duan et al. (our unpublished data) characterized three homologs of the HAK/KT/KUP family from *S. salsa*, and revealed they might play important roles in mediating root K^+ uptake and transport. However, very little is known about AKT1-type channels in *S. salsa*.

The first *AKT1* encoding an inward-rectifying K^+ channel was cloned from Arabidopsis by functional complementation of yeast mutant strains defective in K^+ transport system (Sentenac et al. 1992). Previous research has shown that AKT1 is an important component for both high- and low-affinity K^+ uptake, and *AKT1* genes are expressed primarily in roots, especially in mature epidermis, cortex and endodermis (Ardie et al. 2010; Gierth and Mäser 2007; Hirsch et al. 1998; Lagarde et al. 1996; Rubio et al. 2008; Spalding et al. 1999; Xu et al. 2014). It has also been reported that the transcripts of *AKT1* were regulated by external Na^+ concentrations (Ardie et al. 2010; Boscari et al. 2009; Fuchs et al. 2005; Golldack et al. 2003; Su et al. 2001). In rice, expression of *OsAKT1* was down-regulated and inward K^+ currents mediated by OsAKT1 were significantly reduced in root protoplasts in response to salt

stress (Fuchs et al. 2005). In contrast, the expression of *HvAKT1* in the elongation zone of leaves in barley was induced by salt, probably contributing to the maintenance of K^+ concentration in mesophyll cells under salinity (Boscari et al. 2009). *PutAKT1* transcript levels from *Puccinellia tenuiflora* seemed to be unaffected by the presence of high external Na^+ concentration, and Arabidopsis plants over-expressing *PutAKT1* showed increased K^+ contents and enhanced salt tolerance compared to wild-type plants under salt stress (Ardie et al. 2010). However, the response of *AKT1* to external saline conditions in *S. salsa* remains unknown.

In the present work, the SsAKT1 gene encoding the inward-rectifying K^+ channel was isolated from *S. salsa*, and its function in K^+ transport characterized by yeast complementation assays. Finally, the expression patterns of *SsAKT1* in roots exposed to different KCl or NaCl concentrations were analyzed. The results suggest that SsAKT1 is a potential candidate in mediating K^+ uptake and maintaining K^+ homeostasis under salinity in *S. salsa*.

Materials and methods

Plant materials, growth conditions and treatments

Seeds of *S. salsa* were collected from the side of Chagannuoer Soda Lake in the Inner-Mongolia Autonomous Region, China. Seeds were rinsed three times with distilled water and then germinated at 28 °C on filter paper in the dark for 24 h. Uniform seedlings were transplanted into a plugged hole in plastic containers (5 cm×5 cm×5 cm; 4 seedlings/container) filled with sand and irrigated with modified Hoagland nutrient solution containing 6 mM KNO_3, 1 mM $NH_4H_2PO_4$, 0.5 mM $MgSO_4 \cdot 7H_2O$, 0.5 mM $Ca(NO_3)_2 \cdot 4H_2O$, 60 μM Fe-citrate, 92 μM H_3BO_3, 18 μM $MnCl_2 \cdot 4H_2O$, 1.6 μM $ZnSO_4 \cdot 7H_2O$, 0.6 μM $CuSO_4 \cdot 5H_2O$, 0.7 μM $((NH_4)_6Mo_7)_{24} \cdot 4H_2O$. Solutions were renewed every 3 days. Seedlings were grown in a greenhouse where the temperature was 28 °C/23 °C (day/night), the daily photoperiod was 16/8 h (light/dark; the flux density was approximately 600 μmol/$m^2 \cdot s$) and relative humidity was about 65 %. Three week-old seedlings were used for the following treatments. (i) Plants were treated with modified Hoagland nutrient solution without KNO_3 for 3 d (6 mM KNO_3 was substituted by 3 mM NH_4NO_3) and subsequently 1 or 5 mM KCl were added for 6 h. (ii) After K^+ starvation for 3 d (6 mM KNO_3 substituted by 3 mM NH_4NO_3), plants were treated with additional 0, 0.1, 0.5, 1, 5 or 10 mM KCl for 6 or 48 h. (iii) Plants were treated with modified Hoagland nutrient solution supplemented with 25 or 150 mM NaCl for 6 h. (iv) Plants were treated with modified Hoagland nutrient solution supplemented with additional 0, 25, 50, 100, 150 or 250 mM NaCl for 6 or 48 h. The treatment solutions were changed every day to maintain a constant ion concentration.

Cloning of *SsAKT1*

After K^+ deprivation for 3 d, 3 week-old seedlings were irrigated with modified Hoagland nutrient solution containing 5 mM KCl for 6 h. The root samples were collected and quickly washed three times in distilled water, and dried with filter paper, then immediately frozen in liquid nitrogen and stored at −80 °C until use. Total RNA was extracted using the RNAprep pure plant Kit (TianGen, Biotech Co., Ltd, Beijing, China) following the manufacturer's instructions. First strand cDNA was synthesized from 1 μg of total RNA using an Oligo $(dT)_{18}$ primer and MMLV-reverse transcriptase (Takara, Biotech Co., Ltd, Dalian, China). The partial cDNA fragment was amplified by RT-PCR using a pair of degenerated primers (P1 and P2) corresponding to conserved regions of AKT1-like K^+ channels from other plants (Supplementary Table S1). PCR amplification was programmed at 94 °C for 2 min; 30 cycles of 94 °C for 30 s, 56 °C for 50 s and 72 °C for 50 s; and a final extension at 72 °C for 10 min. PCR products were purified from agarose gels, ligated into the pGEM-T vector (Promega, China) and sequenced by Sangon (China). The 5′- and 3′- ends of *SsAKT1* were obtained with the Rapid Amplification Kit (Invitrogen, USA) according to the instructions and 5′- end specific primers P3, P4, 3′- end specific primers P5, P6 (Supplementary Table S1), respectively. These fragments were assembled to obtain the full-length of the *SsAKT1* cDNA.

Sequence and phylogenetic analysis

A BLAST search was performed on the NCBI platform (http://www.ncbi.nlm.nih.gov/BLAST). The cDNA sequence was analyzed by the DNAMAN 6.0 software. The phylogenetic tree was generated by the MEGA 6.0 software using the maximum-likelihood method and 1000 bootstrap replicates (Tamura et al.

2007). Multiple Sequence alignment was performed using the DNAMAN 6.0 software. The hydrophobicity values were calculated by the program TMpred available at http://www.ch.embnet.orgy/software/TMpred_form.html. The degenerate primers and specific primers were designed with Primer 6.0 software (Premier Biosoft International, Palo Alto, CA, USA).

Real-time quantitative PCR

The reverse transcribed cDNAs were used for real-time quantitative PCR, which was performed on a thermal cycler (ABI PRISM 7500, USA). A specific fragment (136 bp) of *SsAKT1* was amplified with a pair of primers P7 and P8 (Supplementary Table S1). *SsACTIN* (Accession NO. EU429457) was used for RNA normalization, the specific primers of *SsACTIN* that amplified a 111 bp fragment were A1 and A2 (Supplementary Table S1). SYBR Green PCR master mix (Takara, Biotech Co., Ltd, Dalian, China) was used for 20 μL PCR reactions as follow: 95 °C for 30 s, and 40 cycles of 95 °C for 5 s and 60 °C for 34 s. Three independent experiments were conducted and each sample in one independent experiment was assayed three times. The relative expression level (REL) of each sample was estimated according to the following equation as described by Livak and Schmittgen (2001): $REL=2^{-\,ddCt}$, where the ddCt value was the dCt value of *SsAKT1* in each sample minus the dCt value of the calibrator. The dCt value of *SsAKT1* came from the difference between the Ct value of *SsAKT1* and the Ct value of *SsACTIN* in each sample. The dCt value of the calibrator was the mean value from the difference between the Ct value of *SsAKT1* and the Ct value of *SsACTIN* in a sample under control conditions. The Ct value of *SsAKT1* and *SsACTIN* in samples was obtained from the thermal cycler (ABI PRISM 7500, USA).

Plasmid construction

The cDNA fragment containing the open reading frame (ORF) of *SsAKT1* was amplified from roots of *S. salsa* seedlings by RT-PCR with a pair of specific primers P9 and P10 (Supplementary Table S1, *Xba* I and *Xho* I restriction sites underlined). The cDNA fragment containing the ORF of *AtAKT1* and *AtHKT1;1* were amplified from roots of *A. thaliana* seedlings by RT-PCR with a pair of specific primers P11 and P12 (Supplementary Table S1, *Xba* I and *Xho* I restriction sites underlined) and a pair of specific primers P13 and P14 (Supplementary Table S1, *Sma* I and *Hind* III restriction sites underlined), respectively. The resulting products were cloned into a yeast expression vector p416 GPD (Mumberg et al. 1995) by digesting and ligating with corresponding restriction endonuclease, and therefore, generating constructed plasmids p416-SsAKT1, p416-AtAKT1, p416-AtHKT1;1, respectively. All constructs were verified by sequencing.

Yeast complementation assays

The yeast (*Saccharomyces cerevisiae*) strains CY162 (*MATa ura3 his3 his4 trk1Δtrk2Δ*::pCK64) defective in the K^+ transporters TRK1 and TRK2 (Anderson et al. 1992) and G19 (*MATa ade2 ura3 leu2 his3 trp1 ena1Δ::HIS3Δ::ena4Δ* provided by Professor Alonso Rodríguez-Navarro, Universidad Politécnica de Madrid, Madrid, Spain) disrupted in the *ENA1-4* genes encoding Na^+ export pumps (Quintero et al. 1996) were used for yeast complementation assays. Yeast transformations of above constructed plasmids were performed using LiCl as described by Chen et al. (1992). Positive transformants were selected on Ura-selective medium (0.67 % [*w/v*] yeast nitrogen base without amino acids, 0.077 % [*w/v*] DO Supplement-Ura, 2 % [*w/v*] glucose, 100 mM KCl, and 1.5 % [*w/v*] agar), and isolated for subsequent growth experiments.

Yeast growth experiments were performed on arginine-phosphate (AP) medium (8 mM phosphoric acid, 10 mM L-Arginine, 2 mM $MgSO_4$, 0.2 mM $CaCl_2$, 2 % glucose, plus vitamins and trace elements, and 1.5 % [*w/v*] agar, pH=6.5) (Rodríguez-Navarro and Ramos 1984). For growth tests of CY162 transformed with plasmids, AP medium supplemented with three concentrations of K^+ (0.2, 1 and 100 mM) were used. AP medium with added K^+ (1 mM) and supplemented with various concentrations of Na^+ (0, 10, 30 and 50 mM) were used for growth assays of G19 transformed with plasmids. Yeast cells were plated on medium using ten-fold serial dilutions calculated from $OD_{600}=0.6$ to $OD_{600}=0.6\times10^{-3}$.

For kinetic analysis of K^+ uptake in yeast, yeast colonies expressing *SsAKT1* and *AtAKT1* were cultured at 28 °C overnight in 50 mL liquid Ura-selective medium, until the OD_{600} reached 2.5. Then the yeast cells were collected by centrifugation and washed three times in double-distilled water and then resuspended in double-distilled water to an OD_{600} value of 3.0. Yeast cells (100 uL) were transferred into the AP medium

(30 mL) supplemented with 50, 75, 100, 125, 150, 200, 350, 500 or 1000 μM KCl in 50 mL flasks, and shaken at 28 °C. The OD_{600} values were recorded every 1.5 h after the OD_{600} reached 0.2. The slope for each K^+ concentration was calculated according to the linear regression of the growth curves during the logarithmic growth phase. The curve was obtained by applying nonlinear regression analysis using the Michaelis-Menten equation (Horie et al. 2011; Li et al. 2014).

Statistical analyses

Results of *SsAKT1* relative expression levels are presented as means ±SD (n=3) and data analysis was performed by ANOVA using SPSS statistical software (Ver. 13.0, SPSS Inc., Chicago, IL, USA). Duncan's multiple range tests were used to detect differences among means at a significance level of $P<0.05$.

Results

Isolation and characterization of SsAKT1

A fragment of 749 bp was isolated with the degenerate primers P1 and P2 (Supplementary Table S1) by RT-PCR. Nucleotide BLAST search showed that this cDNA fragment shared high homology (73–78 %) with many known *AKT1* genes from other plants, suggesting that a partial putative *AKT1* had been isolated from *S. salsa*. Specific primers (Supplementary Table S1) were further designed based on this fragment and 5′- RACE and 3′- RACE were performed, and a 5′- RACE product of 1081 bp and a 3′- RACE product of 1975 bp were amplified, respectively. Finally, a full-length cDNA of *AKT1* was obtained, which was 3182 bp long and contained a 5′- untranslated region (UTR) of 74 bp nucleotides, a predicted ORF of 2640 bp nucleotides, and a 3′- UTR of 468 bp nucleotides (Supplementary Fig. S1). The deduced amino acid sequence of this AKT1-like protein showed that it contained 879 amino acid residues with estimated molecular mass of 98.8 kDa and a theoretical isoelectric point of 6.55 (data not shown). We therefore designated this gene as *SsAKT1*.

Multiple sequence alignment revealed that SsAKT1 shared high similarity with other AKT1 previously characterized in higher plants, and its amino acid sequence identity to MKT1 from *Mesembryanthemum crystallinum*, VvK1.2 from *Vitis vinifera* and GmAKT1 from *Glycine max* was 67, 62 and 61 %, respectively (Fig. 1). Furthermore, SsAKT1 exhibited all the structural features shared by other plant inward-rectifying K^+ channels (Sentenac et al. 1992; Uozumi et al. 1998), including six transmembrane domains (TM1-TM6), a K^+-selective pore-forming domain (Pore) comprising a TxxTxGYGD motif between TM5 and TM6, a putative cyclic nucleotide-binding domain (cNBD), an ankyrin domain (ANK), a domain rich in hydrophobic and acidic residues (K_{HA} domain) (Fig. 1). Moreover, the plant Shaker family can be divided into five groups (group I-V represented by the Arabidopsis AKT1, KAT1, AKT2, AtKC1 and SKOR, respectively) (Pilot et al. 2003), and phylogenetic analysis showed that SsAKT1 was grouped into GROUP I (AKT1 type inward-rectifying K^+ channel), and formed a clade with the closest relation to the dicotyledons AKT1 homologue MKT1, but was distinct from the cluster of monocotyledonous AKT1 such as PutAKT1 from *Puccinellia tenuiflora* (Fig. 2, Supplementary Fig. S2).

SsAKT1 mediates K^+ uptake in yeast cells

CY162 is a K^+-uptake-deficient yeast mutant deleted in the two K^+ transporters TRK1 and TRK2 (Anderson et al. 1992); Arabidopsis AtAKT1 was able to complement the growth of yeast *trk1*$^{\triangle}$ *trk2*$^{\triangle}$ mutant under low K^+ concentration by endowing the yeast cells with K^+ uptake capacity (Sentenac et al. 1992). To analyze whether SsAKT1 functions in K^+ uptake, we then expressed *SsAKT1* and *AtAKT1* (as a positive control) in CY162 (Fig. 3). CY162 transformed with empty p416 GPD, p416-AtAKT1 and p416-SsAKT1 grew equally well on the control medium containing 100 mM K^+ (Fig. 3). However, CY162 transformed with empty p416 GPD could not grow on the low-K^+ medium containing 0.2 and 1 mM K^+ after incubation for 48 h while expression of *SsAKT1* as well as *AtAKT1* permitted CY162 cells to grow (Fig. 3). Moreover, the growth of CY162 cells transformed with p416-SsAKT1 and p416-AtAKT1 in liquid AP medium supplemented with different K^+ concentration (50–1000 μM) was monitored by measuring OD_{600} values. The data were fitted to Michaelis-Menten equations and K_m values were determined (Fig. 4). Similar to AtAKT1, SsAKT1 could mediate high-affinity K^+ uptake in yeast cells under low K^+ concentrations ($K_{m\ AtAKT1}$=110.9±5.2 μM, R^2=0.99; $K_{m\ SsAKT1}$=120.8±15.8 μM, R^2=0.95) (Fig. 4).

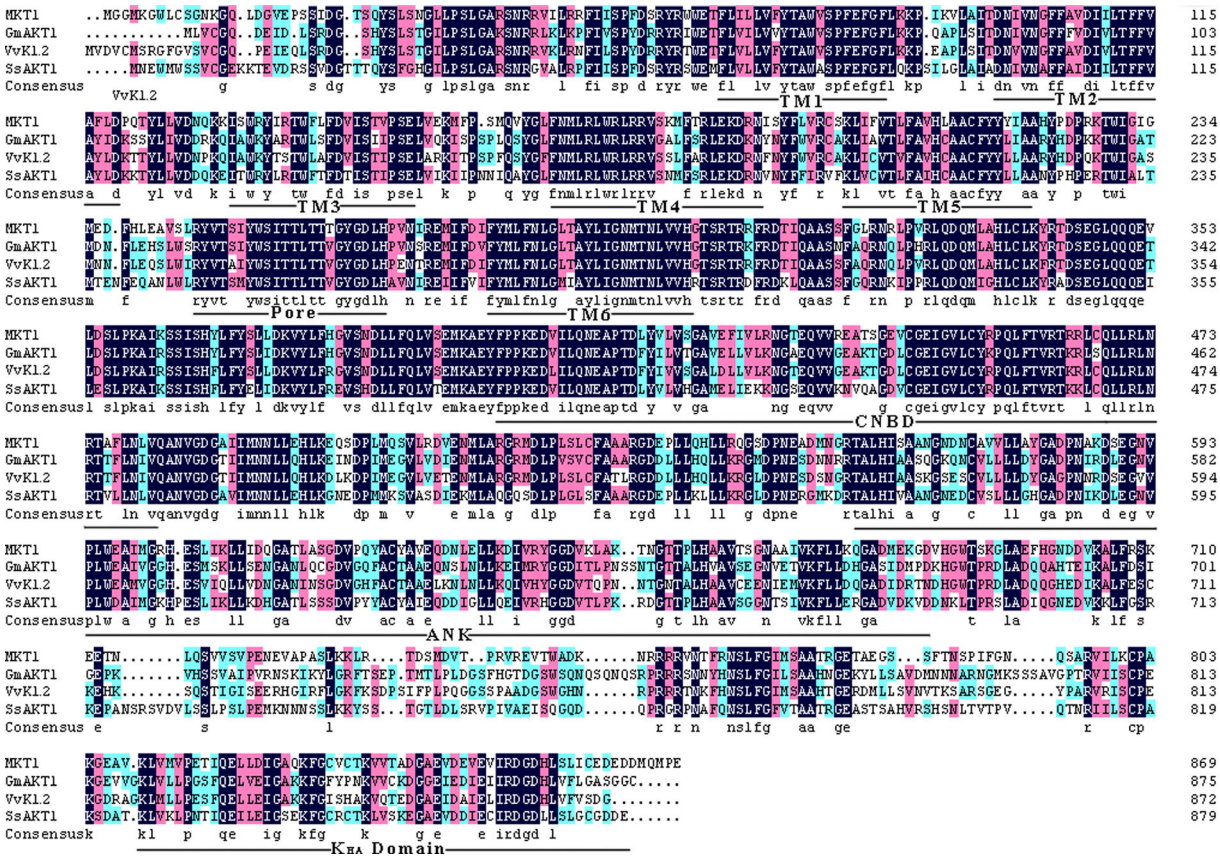

Fig. 1 Sequence alignment of SsAKT1 with other AKT1 from higher plants. Sources of AKT1 and their GenBank accession numbers were as follows: MKT1 (*Mesembryanthemum crystallinum*, AF267753), VvK1.2 (*Vitis vinifera*, FR669116), GmAKT1 (*Glycine max*, XP_003549784). The sequences were aligned with DNAMAN 6.0 software. The six putative transmembrane domains (TM1-TM6) and other domains (Pore, cNBD, ANK and K_{HA} domain) are underlined

To determine whether SsAKT1 could mediate Na^+ uptake, the empty p416 GPD vector, p416-AtAKT1 and p416-SsAKT1 were transformed respectively into a yeast mutant G19 which displayed higher salt sensitivity to Na^+ than the wild-type yeast strain as a result of disruptions in genes *ENA1* to *ENA4* encoding Na^+-extruding ATPase (Quintero et al. 1996). Since AtHKT1;1 conferred increased Na^+ sensitivity on G19 by mediating Na^+ uptake (Uozumi et al. 2000), we used AtHKT1;1 as a positive control for analyzing Na^+ uptake. Growth assays indicated that all the yeast cells grew well on the control medium (0 mM Na^+) (Fig. 5). With the increase of external Na^+ concentration (10–50 mM), as expected, G19 expressing *AtHKT1;1* exhibited Na^+ hypersensitivity compared to control cells (G19 transformed with empty p416 GPD); in contrast, the expression of *SsAKT1* and *AtAKT1* significantly suppressed the salt-sensitive phenotype of G19: yeast cells expressing *SsAKT1* and *AtAKT1* showed better growth than control cells (Fig. 5). It should be noted that the AP medium used in this experiment contained 1 mM K^+, and G19 expressing *SsAKT1* and *AtAKT1* had higher K^+ uptake capacity than control cells since these two proteins could mediate K^+ uptake as shown in Fig. 3. Consequently, compared to control cells, G19 expressing *SsAKT1* and *AtAKT1* could accumulate more K^+ to alleviate Na^+ toxicity, and exhibited better growth even under higher Na^+ concentration (50 mM) (Fig. 5). Our data, therefore, suggested that SsAKT1 could not participate in Na^+ uptake and functioned as a K^+ transporter in yeast cells.

Expression of *SsAKT1* in *S. salsa* under KCl treatments

We investigated tissue-specific expression of *SsAKT1* in *S. salsa* under KCl treatments by real-time quantitative PCR (Fig. 6a). *SsAKT1* was predominantly expressed in roots, barely in leaves and not expressed in stems of

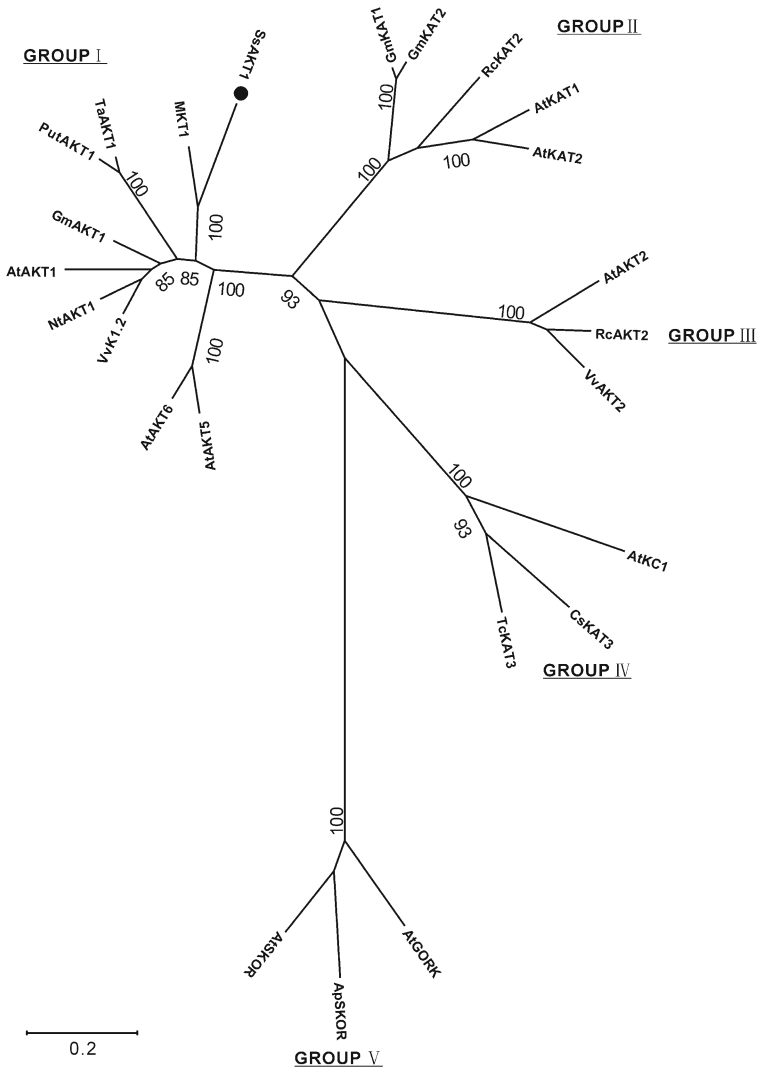

Fig. 2 Phylogenetic groups of SsAKT1 and Shaker K^+ channels from plants. The phylogenetic tree was generated by MEGA 6.0 software using the maximum-likelihood method and 1000 bootstrap replicates. Bootstrap values (as percentages) are indicated at the corresponding nodes. The *scale bar* corresponds to a distance of 10 changes per 100 amino acid positions. SsAKT1 is shown as ●. Sources of Shaker K^+ channels and their GenBank accession numbers are as follows: MKT1 (*Mesembryanthemum crystallinum*, AF267753), VvK1.2 (*Vitis vinifera*, FR669116), GmAKT1 (*Glycine max*, XP_003549784), NtAKT1 (*Nicotiana tomentosiformis*, XP_009619489), PutAKT1 (*Puccinellia tenuiflora*, GU327382), TaAKT1 (*Triticum aestivum*, AF207745), AtAKT1 (*Arabidopsis thaliana*, NM_128222), AtAKT5 (*Arabidopsis thaliana*, NP_194976), AtAKT6 (*Arabidopsis thaliana*, NM_128222), AtAKT2 (*Arabidopsis thaliana*, At4g22200), AtKAT1 (*Arabidopsis thaliana*, At5g46240), AtKAT2 (*Arabidopsis thaliana*, At4g18290), AtKC1 (*Arabidopsis thaliana*, At4g32650), AtSKOR (*Arabidopsis thaliana*, At3g02850), AtGORK (*Arabidopsis thaliana*, At5g37500), VvAKT2 (*Vitis vinifera*, XP_002268924), RcAKT2 (*Ricinus communis*, XP_002529533), RcKAT2 (*Ricinus communis*, XP_002519693), GmKAT1 (*Glycine max*, XP_003541662), GmKAT2 (*Glycine max*, XP_003547208), TcKAT2 (*Theobroma cacao*, EOY29638), CsKAT3 (*Cucumis sativus*, 004162067), ApSKOR (*Alternanthera philoxeroides*, AFO70199). *Open brace* indicates the number substitutions per site

S. salsa. The expression level of *SsAKT1* in roots was significantly induced by 1 and 5 mM KCl (Fig. 6a).

The treatment of K^+ starvation for 3 d significantly induced the expression of *SsAKT1* by approximately 2-fold in roots, compared to that under control condition (6 mM K^+ in medium) (Fig. 6b). After K^+ starvation for 3 d, the expression patterns of *SsAKT1* were analyzed when plants were resupplied with KCl (0.1–10 mM) for 6 and 48 h (Fig. 6c). After 6 h, the transcription level of *SsAKT1* in roots increased significantly

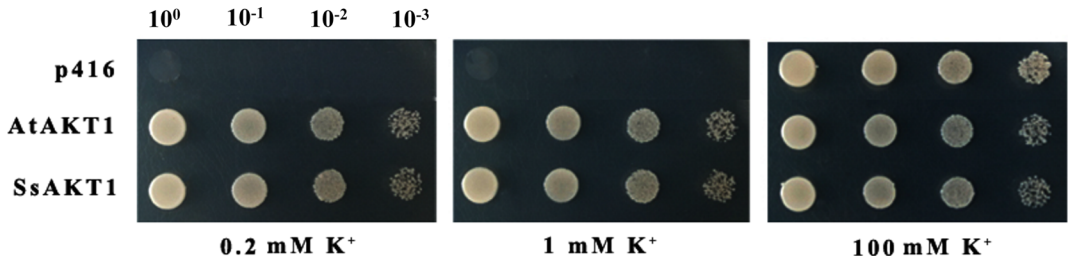

Fig. 3 Complementation of the K^+ uptake deficient *S. cerevisiae* mutant strain CY162 by expressing *AtAKT1*, *SsAKT1* and empty vector p416 GPD. Each yeast cell was plated on minimal AP medium containing three levels of K^+ concentration (0.2, 1 and 100 mM) by ten-fold serial dilutions from OD_{600}=0.6 to OD_{600}= 0.6×10^{-3}. AP medium with 100 mM K^+ was used as control medium, and CY162 expressing *AtAKT1* and p416 GPD were used as positive and negative controls, respectively

when resupplied with 0.1 to 1 mM KCl, peaking at 1 mM KCl by approximately 2.7-fold higher than that under control condition (0 mM KCl), then maintained a stable level under 5 and 10 mM KCl (Fig. 6c). Compared to the control (0 mM KCl), the expression level of *SsAKT1* increased by 62 % at 0.1 mM KCl concentration (which correspond to the range of operation of the high-affinity K^+ uptake system), while significantly increased by 144–174 % under higher KCl conditions (1–10 mM) (under the range of operation of the low-affinity K^+ uptake system) (Fig. 6c). When expression was determined after 48 h, the expression level of *SsAKT1* displayed a slight decrease with the increase of resupplied KCl concentrations (0.1–10 mM) compared with the control (0 mM KCl), but no significant difference was found at different KCl concentrations (0–10 mM) (Fig. 6c), suggesting that *SsAKT1* was only induced within a short period by external resupplied KCl conditions after the plants were subjected to K^+ deprivation.

Expression of *SsAKT1* in *S. salsa* under NaCl treatments

We also explored tissue-specific expression of *SsAKT1* under 25 or 150 mM NaCl for 6 h (Fig. 7a). *SsAKT1* was primarily expressed in roots and significantly induced by 25 mM and 150 mM NaCl; there was very low expression in leaves and with no expression in stems (Fig. 7a).

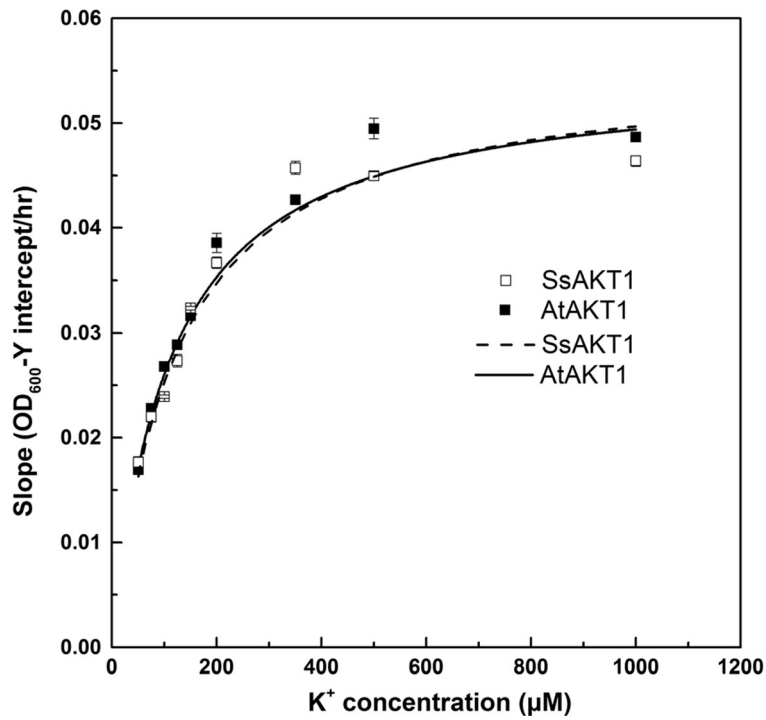

Fig. 4 K^+ uptake kinetic analysis of SsAKT1 and AtAKT1 in *S. cerevisiae* mutant strain CY162. CY162 cells harbouring p416-SsAKT1 or p416-AtAKT1 cDNA were inoculated into liquid AP medium supplemented with 50, 75, 100, 125, 150, 200, 350, 500 and 1000 μM KCl. Growth of the cells was monitored, and the slopes from the linear regression of the growth curves at the logarithmic growth phase of *SsAKT1*-expressing and *AtAKT1*-expressing cells were obtained and plotted. The curve fitting in the graph was performed by a nonlinear regression analysis using the Michaelis-Menten curve-fitting formula with Microcal origin 8.0 software. The data points are shown as means± SE (n=3)

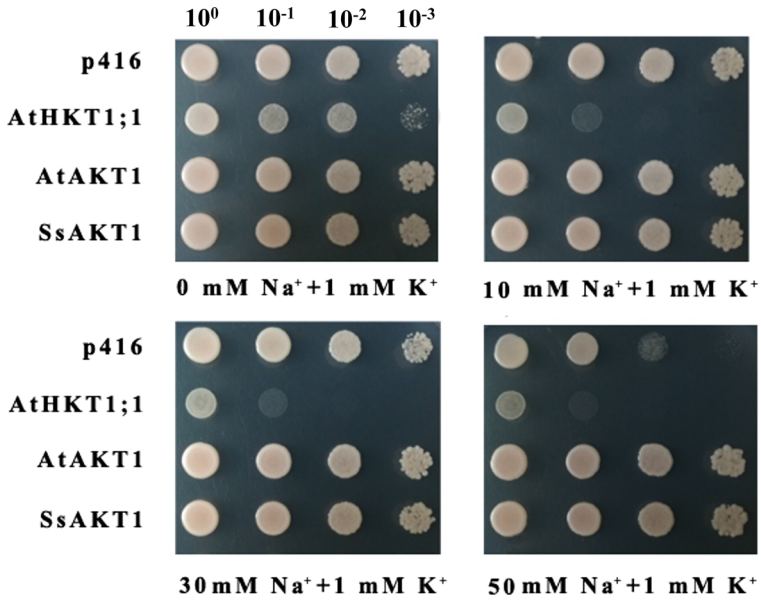

Fig. 5 Na^+-induced growth inhibition of *S. cerevisiae* mutant strain G19 expressing *AtHKT1;1*, *AtAKT1*, *SsAKT1* and empty vector p416 GPD. Yeast cells were plated on minimal AP medium containing 1 mM K^+ and various concentrations of Na^+ (0, 10, 30 and 50 mM) by ten-fold serial dilutions from OD_{600}=0.6 to $OD_{600}=0.6\times10^{-3}$. AP medium without Na^+ was used as control medium, and G19 expressing *AtHKT1;1*, *AtAKT1* and p416 GPD were used as positive and negative controls, respectively

We also analyzed the expression of *SsAKT1* in roots under various NaCl concentrations (0–250 mM) for 6 and 48 h (Fig. 7b). With the increase of NaCl concentrations (0–250 mM), the transcript abundance of *SsAKT1* increased significantly at 6 h, especially in 150 and 250 mM NaCl, where the value was about 1.9-fold and 3.6-fold higher than that under control condition (0 mM NaCl), respectively (Fig. 7b). However, the expression level of *SsAKT1* was down-regulated under NaCl treatments (25–250 mM) at 48 h compared with control (0 mM NaCl) (Fig. 7b). The results indicated that the expression level of *SsAKT1* was only induced by high Na^+ concentration for a short period.

Discussion

SsAKT1 encodes an inward-rectifying K^+ channel in *S. salsa*

Shaker AKT1-type channels from higher plants have been predicted to possess six transmembrane domains

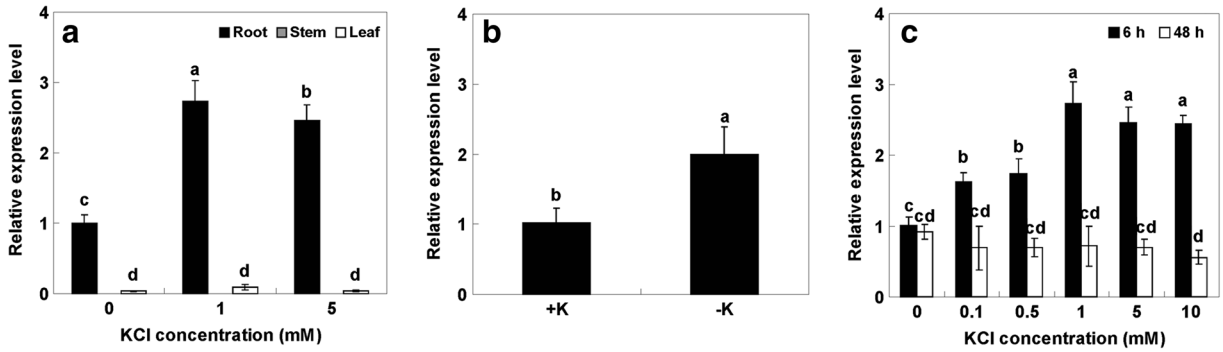

Fig. 6 Expression of *SsAKT1* in *S. salsa* under various KCl concentrations. **a** Tissue specific analysis in roots, stems, and leaves of K^+-starved plants treated with three levels of KCl (0, 1 or 5 mM) for 6 h. **b** The relative expression level of *SsAKT1* mRNA in roots of plants treated with non-K^+ solution for 3 d (−K). The plants grown in normal modified Hoagland medium (+K) were used as control. **c** Real-time qPCR analysis of *SsAKT1* mRNA in roots of K^+-starved plants under various KCl concentrations (0, 0.1, 0.5, 1, 5 or 10 mM) for 6 or 48 h. The 3-week-old plants were deprived of K^+ (see Methods) for 3 d before (**a**) and (**c**) treatments. *SsACTIN* was used as an internal control. The results shown represented qPCR analysis of the cDNA synthesized from three experiments. Values are means ±SD (n=3) and bars indicate SD. *Columns with different letters* indicate significant differences at $P<0.05$ (Duncan's test)

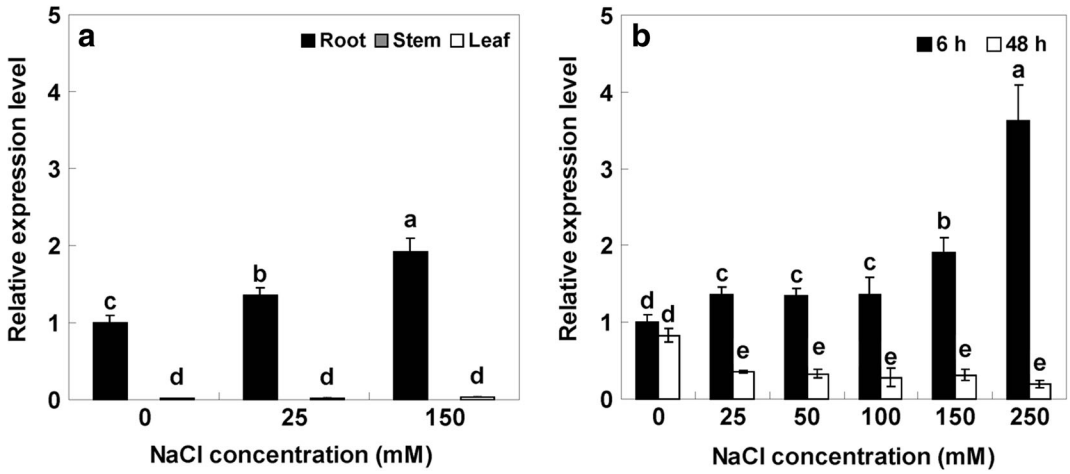

Fig. 7 Expression of *SsAKT1* in *S. salsa* under various NaCl concentrations. **a** Tissue specific analysis in roots, stems, and leaves of 3-week-old plants treated with three concentrations of NaCl (0, 25 or 150 mM) for 6 h. **b** Real-time qPCR analysis of *SsAKT1* mRNA in roots of 3-week-old plants treated with various NaCl concentrations (0, 25, 50, 100, 150 or 250 mM) for 6 or 48 h. *SsACTIN* was used as an internal control. The results shown represent qPCR analysis of the cDNA synthesized from three experiments. Values are means ±SD (n=3) and bars indicate SD. *Columns with different letters* indicate significant differences at $P<0.05$ (Duncan's test)

(TM1-TM6) with a highly conserved pore domain carrying the hallmark TxxTxGYGD/E motif of highly K^+ selective channels and located between TM5 and TM6 (Chérel 2004; Sentenac et al. 1992; Uozumi et al. 1998; Véry and Sentenac 2003). It has been reported that the TM4 domain of AKT1-type channels harbours positively charged amino acids (R and K) and is expected to act as a voltage sensor (Maathuis et al. 1997). The movements of TM4 domain within the membrane could result in channel conformational changes that favour opening or closure of the pore in response to changes in the trans-membrane electrical potential (Véry et al. 2014). Besides, AKT1 from higher plants typically displays a rather short N-terminal domain and a long intracytoplasmic C-terminal region representing more than half of the sequence, which harbours a cNBD domain responsible for subunit interactions, an ANK domain potentially involved in protein-protein interactions, and a K_{HA} domain involved in subunit tetramerization or channel clustering in the membrane (Czempinski et al. 1999; Gambale and Uozumi 2006; Sentenac et al. 1992; Véry and Sentenac 2003). The deduced amino acid sequence of SsAKT1 showed all these typical features of AKT type channels in plants (Fig. 1), suggesting that SsAKT1 has a similar function to other AKT1 proteins from higher plants. SsAKT1 was assigned to Group I (AKT1-type inward-rectifying K^+ channel subfamily) of the plant Shaker family (Fig. 2), which are mainly expressed in roots and involved in K^+ uptake (Pilot et al. 2003). Moreover, the yeast complementation experiments further showed that similar to AtAKT1 in Arabidopsis (Ros et al. 1999; Sentenac et al. 1992), SsAKT1 could mediate K^+ uptake over a wide range of external K^+ concentrations (Figs. 3 and 4); and more interestingly, expression of *SsAKT1* could also enhance salt tolerance of a Na^+-extruding ATPase-deficient yeast strain G19 via endowing the yeast cells with K^+ uptake capacity (Fig. 5), implying that SsAKT1 functioned as a K^+ transporter in yeast. Taken together, our findings showed that *SsAKT1* encoded an AKT1-type inward-rectifying K^+ channel in *S. salsa*.

SsAKT1 might play a crucial role in mediating K^+ uptake in *S. salsa*

Many studies have shown that, *AKT1* is expressed primarily in roots, especially in mature epidermis, cortex and endodermis, and mediates NH_4^+-insensitive K^+ uptake over a wide range of external K^+ concentrations (Bauer et al. 2000; Boscari et al. 2009; Dennison et al. 2001; Hartje et al. 2000; Wang and Wu 2013). In our study, *SsAKT1* was mainly expressed in roots (Figs. 6a and 7a), which was consistent with the observations of *AtAKT1* in *A. thaliana* (Cao et al. 1995; Lagarde et al.

1996), *PutAKT1* in *P. tenuiflora* (Ardie et al. 2010), *VvK1.1* in *V. vinifera* (Cuéllar et al. 2010) and *OsAKT1* in *Oryza sativa* (Fuchs et al. 2005), suggesting the potential role of AKT1 in K^+ uptake in roots (Hirsch et al. 1998; Spalding et al. 1999; Su et al. 2002). The results of heterologous expression in yeast provided further evidences that, SsAKT1 conferred K^+ uptake capacity on the mutant yeast strain CY162 and G19, and thus, rescued growth of CY162 under low K^+ condition (Fig. 3) and enhanced the salt tolerance of G19 (Fig. 5). In wheat, *TaAKT1* mRNA levels were up-regulated in roots in response to withdrawal of K^+ from the growth medium, and K^+ starvation was found to enhance the magnitude and frequency of occurrence of time-dependent inward-rectifying K^+ channel currents, indicating that TaAKT1 might contribute to K^+ uptake in wheat roots under K^+-starvation condition (Buschmann et al. 2000). In the present work, the transcript levels of *SsAKT1* in *S. salsa* roots were also induced significantly by K^+ starvation (Fig. 6b), implying the possible function of SsAKT1 in K^+ uptake under K^+ deficient condition.

Previous research indicated that K^+ uptake in higher plants showed typical dual-affinity (high- and low-) mechanisms, which operated at different external K^+ concentrations (Epstein et al. 1963; Maathuis and Sanders 1994; Wang and Wu 2013). The high-affinity K^+ uptake mechanism mediates K^+ uptake at low external K^+ concentrations (below 0.2 mM), while the low-affinity K^+ uptake mechanism, mediated primarily by K^+ channels, is involved in K^+ uptake at relatively high external K^+ concentrations (above 0.3 mM) (Epstein et al. 1963; Maathuis and Sanders 1994; Schroeder et al. 1994; Wang and Wu 2013). Hartje et al. (2000) found that K^+ inward currents of *Xenopus oocytes* injected with an AKT1 derived from *Solanum esculentum* increased significantly under high K^+ concentration, and concluded it might serve as a low-affinity influx pathway for K^+ into root hair cells. Further evidence that, GhAKT1 of *Gossypium hirsutum* could mediate K^+ uptake from very low K^+ concentration (100 μM), within the range of operation of the high-affinity K^+ uptake system was supplied by Xu et al. (2014). However, study of an *A. thaliana* T-DNA insertion mutant in *AtAKT1* indicated that AtAKT1 contributed to not only low-affinity K^+ uptake, but also high-affinity K^+ uptake in Arabidopsis roots (Gierth and Mäser 2007). In our study, in K^+-deprived plants, the amount of *SsAKT1* transcripts in roots showed a surprisingly strong increase when resupplied with KCl (0.1–10 mM) for 6 h (Fig. 6c). A preceding report (Shao et al. 2014) also showed that, K^+ concentration in leaves and roots of *S. salsa* seedlings previously starved of K^+ increased significantly when resupplied with increasing K^+ concentrations (0.1–6 mM). This coincidence of both increase of *SsAKT1* expression and K^+ accumulation in plants suggests an important role for SsAKT1 in K^+ uptake in roots under different K^+ concentrations. Although there was an increase of *SsAKT1* expression in 0.1 mM K^+ (high-affinity system), *SsAKT1* expression was much higher under higher K^+ concentrations (1–10 mM, the low-affinity K^+ uptake system) (Fig. 6c), implying SsAKT1 was involved in both high- and low-affinity K^+ uptake in *S. salsa*, and might play a greater role in the low-affinity system.

SsAKT1 might be involved in the salt tolerance of *S. salsa*

For most glycophytes, high external Na^+ disturbs intracellular ion homeostasis, leading to cell membrane dysfunction and attenuation of metabolic activity (Blumwald et al. 2000; Volkov and Amtmann 2006). Besides, due to the physicochemical similarities between Na^+ and K^+, Na^+ competes for K^+ absorption sites in root cells, causing reduction of K^+ absorption under high Na^+ concentrations, and resulting in drastic reduction of plant growth and even death (Maathuis and Amtmann 1999; Schachtman and Liu 1999; Schachtman 2000). However, the growth of *Suaeda* species, such as *S. salsa*, *S. glauca, S. fruticosa*, *S. maritima*, is enhanced by external Na^+ treatments (25–400 mM) rather than suppressed, while K^+ concentrations in these plants also increased or remained relatively stable (Khan et al. 2000; Mori et al. 2010, 2011; Song et al. 2009; Wang et al. 2007; Yang et al. 2008; Yeo 1981; Yeo and Flowers 1980).

Some studies showed that the transcription level of genes related to K^+ uptake like *AtAKT1* and *OsAKT1* were down-regulated by salt stress accompanied with a decrease of K^+ absorption (Fuchs et al. 2005; Kaddour et al. 2009). However, *HvAKT1* in barley was induced by salt (100 mM NaCl) in the elongation zone of leaves, probably contributing to the maintenance of K^+ concentration in mesophyll cells during salinity (Boscari et al. 2009). In our study, *SsAKT1* transcript levels in roots increased significantly with the increase of external Na^+ concentration (25–250 mM) for 6 h (Fig. 7b), which

might well explain how K^+ concentration in *S. salsa* could remain relatively constant in shoots and roots with increasing salinity (5–200 mM NaCl) (Zhang 2008). Moreover, our results from heterologous expression studies showed that G19 cells expressing *SsAKT1* exhibited enhanced salt tolerance (Fig. 5), probably because SsAKT1 conferred a higher K^+ uptake capacity in the yeast cells. A similar result was reported by Ros et al. (1999), who showed that expressing *AtAKT1* in a yeast strain 10A (*trk1*$^-$, *ura3*$^-$) defective in high-affinity K^+ uptake system enhanced salt tolerance. Thus, we speculate that the up-regulation of *SsAKT1* expression under saline conditions contributed to mediating significant K^+ uptake in roots from the external medium, providing *S. salsa* with the ability to maintain K^+ homeostasis in the plant under salinity, and ultimately contribute to its salt tolerance.

Besides the Shaker K^+ channels, only a few K^+ transporters in *Suaeda species* have been isolated. In *S. salsa*, the transcript level of *SsHKT1;1*, a gene encoding high-affinity K^+ transporter, was up-regulated by salinity in leaves (Shao et al. 2008); furthermore, transgenic *Arabidopsis thaliana* plants overexpressing *SsHKT1;1* exhibited increased shoot K^+ concentration and enhanced salt tolerance, suggesting that SsHKT1;1 was involved in salt tolerance by taking part in the maintenance of K^+ nutrition (Shao et al. 2014). Duan et al. (our unpublished data) cloned HAK/KT/KUP family members *SsHAK2*, *SsHAK5* and *SsHAK6*, and found SsHAK5 could also improve the salt tolerance of G19 by conferring K^+ uptake capacity. These K^+ transporters and SsAKT1 might cooperate to maintain K^+ homeostasis under salt conditions in *S. salsa*.

In conclusion, SsAKT1 gene encoding the inward-rectifying K^+ channel in *S. salsa*, a potential candidate to mediate both high- and low-affinity K^+ uptake across different K^+ concentration ranges, and likely plays an essential role in salt tolerance of *S. salsa* by contributing to efficient K^+ uptake under saline conditions.

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